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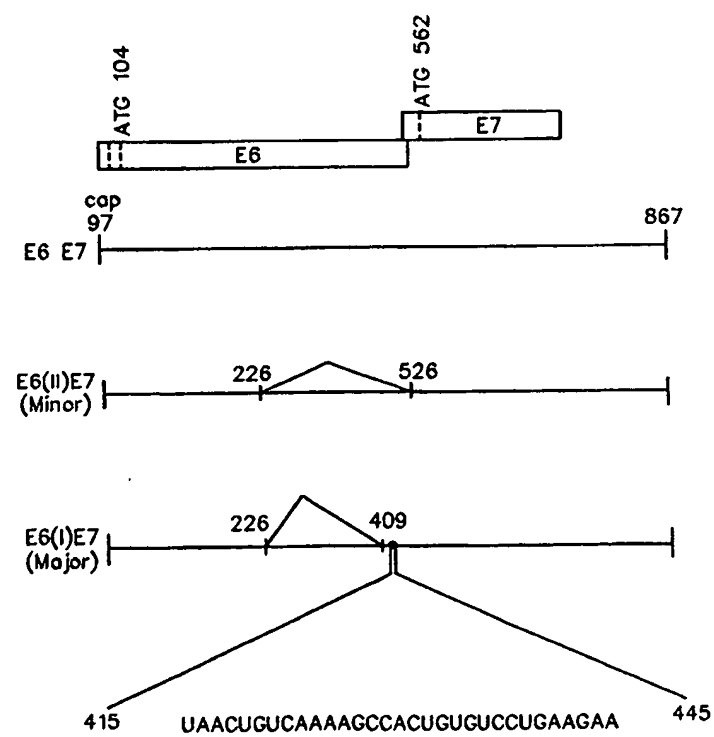
(54) Title: HUMAN PAPILLOMA VIRUS INHIBITION BY ANTI-SENSE OLIGONUCLEOTIDES

(57) Abstract

Antisense oligonucleotides and analogs thereof having phosphorothioate backbone structure and sequences complementary to nucleotides contained with residues 415 to 445 of human papilloma virus 16 (HPV-16). These antisense oligonucleotides and oligonucleotide analogs are used to inhibit HPV-16 expression and to inhibit the growth of cervical tumors.



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HUMAN PAPILLOMA VIRUS INHIBITION BY ANTI-SENSE OLIGONUCLEOTIDES

Field of the Invention

The present invention relates to the use of antisense oligonucleotides to inhibit a Human Papilloma virus (HPV), and specifically relates to use of antisense oligonucleotides specific for nucleotides 415 to 445 of the DNA sequence of HPV-16.

Background of the Invention

Papilloma viruses are small DNA viruses that induce the hyperproliferation of epithelial cells. Approximately 70 different genotypes of human papilloma virus (HPV) have been isolated. Some HPV genotypes (e.g., 1, 2, 4, and 7) have been associated with human benign squamous papillomas (warts and condylomas) and others (e.g., 16 and 18) have been associated with human neoplastic and preneoplastic lesions (DiPaolo, et al., 1993, *Crit. Rev. Oncogen.* 4:337-360).

HPV-16 has been associated with a variety of clinical conditions in both women and men. In women, HPV-16 is frequently associated with latent infections, benign and premalignant cervical lesions (dysplasias/CIN) and half of invasive cervical carcinomas. Cervical cancer, which kills at least 500,000 women worldwide each year, proceeds through progressive cellular changes from benign condylomata to high-grade dysplasias/CIN before developing into an invasive cancer. In men, HPV-16 is associated with subclinical macular or clinical papular lesions. One such lesion, Penile Bowenoid papulosis, resembles cervical carcinoma *in situ*. Detection and treatment of these lesions costs over five billion health care dollars annually in the United States.

HPV-16 has been associated with over half of the invasive cervical carcinomas diagnosed worldwide and with many cell lines derived from cervical carcinomas. HPV-16 expression causes benign proliferation and efficiently immortalizes cultured human epithelial cells, including cervical keratinocytes (DiPaolo, et al., 1993, *Crit. Rev. Oncogen.* 4:337-360; Zur Hausen & de Villiers, 1994, *Annu. Rev. Microbiol.* 48:427-447; Schiffman, 1995, *J. Natl. Cancer Inst.* 87:1345-1347). Two HPV-16 genes, E6 and E7, and their gene products are required to immortalize human keratinocytes and are a hallmark of cervical carcinoma (Hawley-Nelson et al., 1989, *EMBO J.* 8:3905-3910; Phelps et al., 1988, *Cell* 53:539-547; Viallet et al., 1994, *Exp. Cell Res.* 212:36-41; Yokoyama et al., *Obstet. Gynecol.* 83:197-204). The E6 and E7 proteins bind to other gene products (p53 and Rb tumor suppressors) to disrupt control of cell division and proliferation, leading to transformation (Scheffner et al., 1990, *Cell* 63:1129-1136; Zerfass et al., *J. Virol.* 69:6389-6399)

Surgery is commonly used for treatment of high-grade lesions due to the lack of effective alternatives. Cervical laser ablation therapy, however, does not in the long term influence the natural history of cervical human papillomavirus-associated diseases in women. Interferons have not proved an effective antiviral or anticancer treatment. Chemotherapy (e.g., cisplatin, alone or combined with other chemotherapy agents such as 5-fluorouracil) has generally not proved to be effective in treatment of many cervical cancers. Moreover, most chemotherapeutic agents are cytotoxic, leading to toxic side effects and the development of multiple drug resistance. Therefore, there

is a need for reagents than can specifically inhibit the growth of HPV-associated tumor cells, while avoiding serious toxic reactions.

HPV-specific treatments in the form of cleavage of HPV-specific RNA with ribozymes and inhibition by HPV-specific antisense oligonucleotides have been suggested (PCT International Patent Application WO 95/31552; DiPaolo, et al., 1993, *Crit. Rev. Oncogen.* 4:337-360; Steele, et al., 1993, *Cancer Res.* 53:2330; Storey, et al., 1991, *Nuc. Acids Res.* 19(15):4109). Ribozymes are small catalytic RNA molecules that can hybridize to and cleave a complementary RNA target (Cech, 1988, *JAMA* 260:3030-3034). Ribozymes having a "hairpin" motif have been found to be more efficient than the "hammerhead" motif (Hampel & Tritz, 1989, *Biochem.* 28:4929-4933; Hampel, et al., 1990, *Nuc. Acids Res.* 18:299-304) and "hairpin" ribozymes have been used to cleave viral targets, including the human immunodeficiency virus (HIV-1) and HPV (Ojwang, et al., 1992, *Proc. Natl. Acad. Sci. USA* 89, 10802-10806; Yu, et al., 1993, *Proc. Natl. Acad. Sci. USA* 90:6340-6344; PCT International Patent Application WO 95/31552).

Antisense RNA and oligonucleotides hybridize to complementary mRNA, thus blocking translation and promoting the activity of endogenous RNase H to cleave the mRNA (Walder, 1988, *Genes Dev.* 2:502-504; Cohen, 1991, *Antisense Res. Dev.* 1:191-193). Although antisense RNA and oligonucleotides should be specific for their target sequence, nonspecific toxicity has been observed (Henry et al., 1997, *Toxicol.* 116:77-88; Henry et al., 1997, *Anticancer Drug Des.* 12:1-14). First-generation antisense phosphorothioates, whose nucleotide backbones carry sulfur atoms to slow intracellular degradation were often ineffective because of their inability to enter cells or to complement the target mRNA, but improved second generation phosphorothioate antisense therapies, referred to as "mixed backbone oligonucleotides" and "end-modified chimerics" that carry 2'-*O*-methylribonucleoside moieties have proven effective in clinical trials (Monia et al., 1996, *Nature Medicine* 6:668-675; Roush, 1997, *Science* 276:1192-1193; Agrawal et al., 1997, *Proc. Natl. Acad. Sci. USA* 94:2620-2625; Agrawal, 1996, *TIBTECH* 14:3-14).

Antisense inhibition of HPV-18 E6 and E7 expression in cell lines (C4-1 and HeLa) resulted in a significant decrease in growth rate with continuous addition of oligonucleotide (Steele, et al., 1993, *Cancer Res.* 53:2330-2337). Similar results have been observed in cells transfected with recombinant vectors (von Knebel Doeberitz & Gissmann, 1987, *Hamatol. Bluttransfus.* 31:377-279; Hamada et al., 1996, *Gynecol. Oncol.* 63:219-227).

The present invention discloses oligonucleotide sequences and methods of antisense therapy using antisense oligonucleotides defined by selected HPV-16 complementary sequences.

Summary of the Invention

According to the present invention, antisense oligonucleotides that specifically bind to a human papilloma virus-16 (HPV-16) sequence include sequences complementary to viral sequences between viral nucleotide 415 and 445.

One aspect of the present invention relates to analogs of antisense oligonucleotides comprising oligonucleotide sequences complementary to SEQ ID NO: 2 or SEQ ID NO: 3, wherein the analogs are phosphorothioate antisense oligonucleotides in which at least one phosphodiester bond is replaced with a phosphorothioate bond, mixed backbone antisense oligonucleotides in which at least one phosphodiester bond is

replaced with a phosphorothioate bond and at least one phosphodiester bond is replaced with a 2'-*O*-methylnucleoside phophodiester bond, end-modified analogs in which at least one end has a 2'-*O*-methylnucleotide moiety, methylphosphonates, phosphoramidites, phosphorodithioates, or N3'→P5'-phosphoramidites.

Another aspect of the present invention relates to analogs of antisense oligonucleotides comprising sequences of SEQ ID NO:6, SEQ ID NO:7, or SEQ ID NO:17, wherein the analogs are phosphorothioate oligonucleotides in which at least one phosphodiester bond is replaced with a phosphorothioate bond, mixed backbone oligonucleotides in which at least one phosphodiester bond is replaced with a phosphorothioate bond and at least one phosphodiester bond is replaced with a 2'-*O*-methylnucleoside phophodiester bond, end-modified oligonucleotides in which at least one end has a 2'-*O*-methylnucleotide moiety, methylphosphonates, phosphoramidites, phosphorodithioates, or N3'→P5'-phosphoramidites.

Another aspect relates to analogs of antisense oligoribonucleotides of SEQ ID NO:4, SEQ ID NO:5 or SEQ ID NO:16, wherein the analogs are oligoribonucleotide phosphorothioates, 2'-*O*-alkyl oligoribonucleotide phosphorothioates or 2'-*O*-methylribonucleotide methylphosphonates. Yet another aspect of the invention relates to an antisense therapeutic composition comprising an oligonucleotide sequence of SEQ ID NO: 9 and a pharmaceutically acceptable carrier.

Therapeutic compositions include any of the foregoing analogs and a pharmaceutically acceptable carrier. The therapeutic compositions can also include a ribozyme containing sequences complementary to SEQ ID NO: 2 or SEQ ID NO: 3.

Yet one more aspect of the present invention relates to a method of preventing transformation of a living cell by HPV. The method includes providing an antisense therapeutic composition as is described above, providing a living cell capable of being transformed by HPV, transfecting the living cell with the antisense therapeutic composition, and maintaining the viability of the living cell for sufficient time to inhibit expression of HPV gene E6.

Still another aspect of the present invention is a method of preventing transformation of a living cell by HPV. This method includes providing one or more antisense oligonucleotides having sequences complementary to SEQ ID NO: 2 or SEQ ID NO: 3, providing a living cell capable of being transformed by HPV, transfecting the one or more antisense oligonucleotide into the living cell, and maintaining the viability of the living cell for sufficient time to inhibit expression of HPV gene E6. The living cell can be a human keratinocyte, a human cervical cell, or other living cell.

An additional aspect of the invention relates to a method for inhibiting expression of HPV gene E6 in a living cell comprising the steps of: providing one or more antisense oligonucleotides having sequences complementary to SEQ ID NO: 2 or SEQ ID NO: 3; providing a biological sample comprising living cells capable of being infected with HPV; transfecting said one or more antisense oligonucleotides into said living cells; and maintaining the viability of said living cells for sufficient time to allow inhibition of HPV E6 gene expression to occur. The method can also include repeating the transfecting and maintaining steps. The living cell can be a human keratinocyte, a human cervical cell, or other living cell. The step of providing antisense oligonucleotides can include administering antisense oligonucleotides to a living organism by subcutaneous (s.c.), intraperitoneal (i.p.) or intravenous (i.v.) injection, or by painting the antisense oligonucleotides onto the biological sample *in situ*.

An additional aspect of the invention relates to a method of inhibiting expression of HPV gene E6 in a living cell. This method includes the steps of: providing one or more antisense oligonucleotide analogs having sequences of SEQ ID NO:6, SEQ ID NO:7, or SEQ ID NO:17, wherein the one or more antisense oligonucleotide analogs are phosphorothioate oligonucleotides in which at least one phosphodiester bond is replaced with a phosphorothioate bond, mixed backbone oligonucleotides in which at least one phosphodiester bond is replaced with a phosphorothioate bond and at least one phosphodiester bond is replaced with a 2'-*O*-methylnucleoside phophodiester bond, in which at least one end has a 2'-*O*-methylnucleotide moiety, methylphosphonates, phosphoramidites, phosphorodithioates, or N3'→P5'-phosphoramidites; providing a biological sample comprising living cells capable of being infected with HPV; transfecting said antisense oligonucleotide analogs into said living cells; and maintaining the viability of said living cells for sufficient time to allow inhibition of HPV E6 gene expression to occur.

The present invention also includes another aspect which is a method for inhibiting expression of HPV gene E6 in a living cell comprising the steps of: providing one or more antisense oligoribonucleotide analogs of oligoribonucleotides of SEQ ID NO:4, SEQ ID NO:5 or SEQ ID NO:16, wherein the analogs are oligoribonucleotide phosphorothioates, 2'-*O*-alkyl oligoribonucleotide phosphorothioates or 2'-*O*-methylribonucleotide methylphosphonates; providing a biological sample comprising living cells capable of being infected with HPV; transfecting said antisense oligoribonucleotide analogs into said living cells; and maintaining the viability of said living cells for sufficient time to allow inhibition of HPV E6 gene expression to occur.

The present invention also provides a method for inhibiting the growth of cervical tumors, comprising the step of contacting the tumors with one or more analogs of antisense oligonucleotides comprising oligonucleotide sequences complementary to SEQ ID NO:2 or SEQ ID NO:3, wherein the one or more analogs are phosphorothioate antisense oligonucleotides in which at least one phosphodiester bond is replaced with a phosphorothioate bond, mixed backbone antisense oligonucleotides in which at least one phosphodiester bond is replaced with a phosphorothioate bond and at least one phosphodiester bond is replaced with a 2'-*O*-methylnucleoside phophodiester bond, end-modified analogs in which at least one end has a 2'-*O*-methylnucleotide moiety, methylphosphonates, phosphoramidites, phosphorodithioates, or N3'→P5'-phosphoramidites.

Yet another embodiment of the invention is the use of one or more analogs of antisense oligonucleotides comprising oligonucleotide sequences complementary to SEQ ID NO:2 or SEQ ID NO:3, wherein the one or more analogs are phosphorothioate antisense oligonucleotides in which at least one phosphodiester bond is replaced with a phosphorothioate bond, mixed backbone antisense oligonucleotides in which at least one phosphodiester bond is replaced with a phosphorothioate bond and at least one phosphodiester bond is replaced with a 2'-*O*-methylnucleoside phophodiester bond, end-modified analogs in which at least one end has a 2'-*O*-methylnucleotide moiety, methylphosphonates, phosphoramidites, phosphorodithioates, or N3'→P5'-phosphoramidites, for treatment of a cervical tumor.

Brief Description of the Drawings

FIG. 1 is a diagram of the HPV-16 E6/E7 target sites showing the overlaps in the E6/E7 mRNA and the ATG sites of E6 (at nt 104) and of E7 (at nt 562), the primary mRNA transcript having a cap at nt 97 and terminating

at nt 867, a minor form of processed E6/E7 mRNA ("E6(II)E7") spliced at nt 226 and 526, and a major form of processed E6/E7 mRNA ("E6(I)E7") spliced at nt 226 and 409, and the relative position of the antisense target sequence between nt 415 and 445. The complete HPV-16 sequence (GenBank Accession No. K02718) is presented in SEQ ID NO:1 and the antisense target sequence between nt 415-445 occurs therein.

FIG. 2 is a diagram of a hairpin ribozyme for cleavage of HPV-16 E6/E7 mRNA with an optimized helix 1, comprising 8 bp, designed to cleave HPV-16 after position 434 ("cleavage site" indicated by the diagonal line), showing the sequences of the ribozyme (in capital letters) and the substrate (in lower case letters), the regions of base pairing between target substrate (nt 430-445) and ribozyme ("helix 1" and "helix 2"), and the regions of base pairing predicted for the hairpin portion of the ribozyme ("helix 3" and "helix 4").

FIG. 3 shows a gel of ribozyme products of the R434 and R434i ribozymes produced by in vitro transcription and incubated with substrate RNA at a 1:2 molar ratio; the arrows at the right show the positions of the uncleaved substrate, and the 3' and 5' fragments produced by substrate cleavage.

FIG. 4 diagrams the cytomegalovirus promoter/enhancer ("CMV") expression plasmids containing the HPV-16 E6/E7 genes only ("pCR16HH"), and with the *cis*-acting R434 ribozyme or R434i inactive ribozyme coding sequences ("pCR16E6/E7Rz" and "pCR16E6/E7Rzi" respectively).

FIG. 5 shows a graph of cell growth of normal human keratinocytes (HKc) (cell count on the Y-axis) over 2 to 7 days in culture (X-axis) for HKc transfected with the control plasmid, pCR16HH (—○—), the active ribozyme construct, pCR16E6/E7RZ (—□—) and the inactive ribozyme construct, pCR16E6/E7RZi (—△—).

FIG. 6 shows an agarose gel separation of the products of a RT-PCR assay specific for HPV-16 E6/E7 mRNA. HKc were transfected with the E6/E7 construct without any ribozyme sequences (pCR16HH), the active ribozyme construct (pCR16E6/E7RZ), and the inactive ribozyme construct (pCR16E6/E7RZi); the negative "Control" is a RT-PCR reaction run without reverse transcriptase. The lower panel shows that all cells produced the 661 bp β-actin band; the upper panel shows the 492 bp uncleaved E6/E7 transcript band and an internal control band of 326 bp.

FIG. 7 is a graph showing the cell counts (Y-axis) for HKc transfected with the E6/E7 construct without any ribozyme sequences (pCR16HH, left bar), the active ribozyme construct (pCR16E6/E7RZ, middle bar), and the inactive ribozyme construct (pCR16E6/E7RZi, right bar) at 8 weeks of growth following transfection.

FIG. 8 is a graph showing inhibition of growth of CasKi cervical tumor cells in culture. Anti-E6, M4 and M7 oligodeoxynucleotides were phosphorothioated and 50 μM was applied to CasKi cervical carcimona cells for 72 hours. Cell growth was estimated by the colorimetric quantitation of Br-dUTP incorporation. Similar results were obtained for QGU cervical carcinoma cells.

Detailed Description of the Preferred Embodiments

The present invention provides antisense oligonucleotides, oligoribonucleotides and analogs thereof, for inhibiting the expression of HPV-16 E6 and E7 genes which are necessary for viral replication. Because the E6 and E7 genes overlap and produce mRNA molecules that overlap the two genes (as shown in FIG. 1), the E6 and E7 genes together and their mRNA overlapping transcripts will be generally referred to herein as E6/E7 genes and E6/E7

mRNA, respectively. These antisense molecules bind to E6/E7 mRNA in the cell, prevent mRNA translation and promote mRNA degradation by intracellular RNase H. These molecules inhibit the growth of cervical tumor cell lines *in vitro*, and inhibit cervical tumor growth *in vivo* in nude mice.

In the course of characterizing ribozymes that cleave E6/E7 mRNA, ribozymes that are inactive because of changes to the hairpin structure of the ribozyme were found to inhibit cell proliferation *in vitro* showing that the ribozymes were, in fact, acting at least in part as antisense inhibitors. That is, even in the absence of ribozyme-mediated cleavage of E6/E7 mRNA, the introduced ribozyme sequences that contain antisense sequences directed to E6/E7 target sequences were capable of limiting the amount of full-length E6/E7 transcripts. These antisense sequences are the basis for antisense oligonucleotides having modified backbone structure for use as antisense therapeutics.

Ribozyme Constructs and Activity

Potential hairpin ribozyme target sites were identified in the HPV-16 gene sequence for E6/E7 contained within the complete HPV-16 sequence (SEQ ID NO:1), by using a computer program (e.g., GCG DNA Analysis Package, Genetics Computer Group, Madison, WI) to search for 5'-GUC-3' motifs. Several potential cleavage sites were identified in E6 (nt 419, 434, 491, 503 and 514) and E7 (nt 679), and synthetic ribozymes covalently linked to sequences complementary to 15 or 16 nt sequences surrounding the identified potential cleavage sites were produced. The initially synthesized ribozymes were based on the structures of the negative strand of satellite RNA from the tobacco ringspot virus (-sTRSV) (Hampel & Tritz, 1989, *Biochem.* 28:4929-4933; Haseloff & Gerlach, 1989, *Gene* 82:43-52). Of these synthetic ribozymes, only those having sequences complementary to the E6 sites at nt 419 and 434 were found to significantly cleave substrate RNA (greater than 60% when mixed at a 1:2 molar ratio of ribozyme to substrate RNA). Improved ribozymes were then synthesized for these particular target sequences, in which modifications were introduced in helix 4 (based on the findings of Anderson et al., 1994, *Nucl. Acids Res.* 22:1096-1100). An improved ribozyme structure having a sequence complementary to target sequence of nt 430-445 of E6 mRNA is shown in FIG. 2. Using cell free reactions to optimize the length of helix 1 (in the hybrid formed between the target sequence and the ribozyme complementary sequence), the optimum sequences in the target were those corresponding to the HPV-16 sequence from nt 415-429 (UAACUGUCAAAAGCC; SEQ ID NO:2) and nt 430-445 (ACUGUGUCCUGAAGAA; SEQ ID NO:3). Based on these target sequences, antisense oligonucleotides interacting with these target sequences have the following sequences: GGCUUUUAGAAGUUA (SEQ ID NO:4) and UUCUUCAGAGAACAGU (SEQ ID NO:5), for antisense RNA complementary to nt 415-429 and to nt 430-445 of E6, respectively; and GGCTTTTAGAAGTTA (SEQ ID NO:6) and TTCTTCAGAGAACAGT (SEQ ID NO:7), for antisense DNA complementary to nt 415-429 and to nt 430-445 of E6, respectively.

The ribozyme coding sequences were synthesized and cloned using standard procedures. The coding sequences were synthesized in an automated DNA synthesizer (Expedite 8900, Perseptive Biosystems, Framingham, MA) and cloned into a plasmid (pBluescipt KS vector, Strategene, La Jolla, CA). Ribozyme coding sequences were cloned in *cis* to complete E6/E7 gene sequences in another plasmid; the E6/E7 and ribozyme sequences were PCR amplified using standard procedures and cloned into another vector (pCR3.1, Invitrogen Corp., San Diego, CA) to

produce plasmids capable of transcribing ribozymes and target sites in *cis*. Plasmids were linearized and purified using standard procedures (restriction digestion and QIAquick column, Qiagene Inc., Chatsworth, CA) and 1 μg of linear DNA template was incubated with T3 or T7 RNA polymerase, rNTP and α-32P-UTP (Amersham Life Sciences, Arlington Heights, IL) to produce ribozyme and/or RNA substrate using standard procedures (e.g., as provided by Ambion Inc., Austin, TX). Target RNA was gel purified (6% polyacrylamide/7M urea gel) before use by standard methods.

The active ribozyme that is specific for nt 430-445, designated R434, consists of the ribozyme sequence diagrammed in FIG. 2 (SEQ ID NO:8; UUCUUCAGAGAACAGUACCAGAGAAACACACGGACUUCGGUCCGUGGUAUAUUACCUGGUA). An inactive ribozyme in which the A_{24}, A_{25} and A_{28} residues of SEQ ID NO:8 have been replaced with C, G and U, respectively, is referred to as R434i and consists of the ribozyme sequence of SEQ ID NO:9 (UUCUUCAGAGAACAGUACCAGAGCGUCACACGGACUUCGGUCCGUGGUAUAUUACCUGGUA).The active ribozyme that contains sequences complementary to E6 nt 415-430 is referred to as R419 and consists of GGCUUUUAGAAGUUAACCAGAGAAACACACGGACUUCGGUCCGUGGUAUAUUACCUGGUA(SEQ ID NO:10).

In Vitro Ribozyme Activity

Ribozyme activity was measured *in vitro* initially at 37°C in reaction buffer (40 mM Tris-HCl, pH 7.5, 12 mM $MgCl_2$, 2 mM spermidine) containing 25 nM ^{32}P-labeled ribozyme and 50 nM ^{32}P-labeled substrate for 60 min; complete characterization was done using similar reactions except that 1 nM ^{32}P-labeled ribozyme and 30 nM ^{32}P-labeled substrate (1:30 molar ratio) were incubated for 180 min. Ribozyme expression from linear or covalently-closed templates was accomplished by incubating 1 μl of an *in vitro* transcription reaction (described above) with 10^6 cpm of ^{32}P-labeled target RNA in 10 μl of reaction mixture. Reactions were stopped by freezing on dry ice; samples were denatured in loading buffer (80% formamide, 0.01% bromophenol blue, 0.01% xylene cyanol) at 65°C for 10 min and separated by gel electrophoresis (6% polyacrylamide/7M urea gel) using standard methods. Dried gels were exposed to radiographic film (BM-2, Kodak Corp., Rochester, NY) and bands intensities indicative of uncleaved and/or cleaved RNA substrate were quantified using a Phosphorimager 425 (Molecular Dynamics, Sunnyvale, CA).

Ribozymes R419 and R434 were active in the *in vitro* ribozyme reactions in which there was 30-fold excess substrate. The R419 ribozyme had a calculated K_m of 0.098 μM and a k_{CAT} of 0.18 min^{-1}; the R434 ribozyme had a calculated K_m of 0.021 μM and a k_{CAT} of 0.08 min^{-1}. The catalytic efficiency (k_{CAT}/K_m) of R434 (3.81 μM^{-1}) was twice as high as that of R419 (1.84 μM^{-1}). These results show that the antisense oligonucleotide sequences (SEQ ID NO:4 and SEQ ID NO:5) contained in ribozymes R419 and R434 are capable of specifically binding to the HPV-16 target sequences. By analogy, DNA antisense oligonucleotide sequences (SEQ ID NO:6 and SEQ ID NO:7) are equally capable of binding to the HPV-16 target sequences in E6.

The R434i ribozyme is changed in the ribozyme hairpin but contains the same target-recognition site as the R434 ribozyme. These changes abolished catalytic activity of the R343i ribozyme *in vitro* as shown in FIG. 3, in

which aliquots of an *in vitro* reaction were analyzed at 0, 15, 30 and 60 min. for R434 and R434i ribozyme reactions.

In Vivo Ribozyme Activity in Cultured Human Keratinocytes

The R434 and R434i ribozyme sequences were cloned into plasmids for transfection into cells for measurement of *in vivo* activity. Plasmid pCR16-E6/E7RZ contains the E6/E7 gene sequences linked in *cis* to the 5' end of enzymatically-active ribozyme R434 sequence, and plasmid pCR16-E6/E7RZi contains the E6/E7 gene sequences linked in *cis* to the 5' end of enzymatically-inactive ribozyme R434i sequence. In both plasmids, the constructs were under the control of the cytomegalovirus (CMV) promoter/enhancer sequences as diagramed in FIG. 4. Control plasmids ("pCR16HH") contained the E6/E7 gene sequences under the control of the CMV promoter/enhancer sequences but without any ribozyme sequences.

Normal human keratinocytes (HKc) from neonatal foreskins were cultured in MCDB151-LB medium using standard methods (Pirisi et al., 1988, *Carcinogen.* 9:1573-1579) and transfected with 10 μg of plasmid DNA using standard lipofection methods (Lipofectin, Life Technologies Inc., Gaithersburg, MD; Alvarez-Salas et al., 1995, *Cancer Lett.* 91:85-92). Transfected cells were grown in the presence of 200 μg/ml of G418 for two weeks (or four days for immortalization studies) and growth rates were determined in standard six-well plates (10^6 cells/well) in triplicate; cells were counted at the end of the incubation period (Coulter Counter ZM, Coulter Electronics Inc., Hialeah, FL).

Following transfection of HKc with the plasmid constructs, cell growth was assayed at 2 to 7 days post-transfection (shown in FIG. 5). HKc transfected with the active ribozyme construct (pCR16E6/E7RZ, –□–) grew significantly slower than cells transfected with the control plasmid (pCR16HH, –○–) or the inactive ribozyme (pCR16E6/E7RZi, –△–) construct. The latter two were capable of expressing the E6/E7 gene products, whereas the active ribozyme would have limited E6/E7 gene expression. To confirm this, a reverse transcription-polymerase chain reaction (RT-PCR) assay was performed to detect the products of ribozyme cleavage.

The RT-PCR assay was performed as follows. Total RNA was purified from the cultured cells (Rneasy Kit, Qiagen) using standard methods. HPV-16 E6/E7 cDNA was produced from 1 μg of total RNA using standard methods (Superscript II One Shot Kit, Life Technologies). To produce differential sized bands for cleaved and uncleaved E6/E7 mRNA, the upper PCR primers were SEQ ID NO:11 (CAGCAATACAACAAACCG) and SEQ ID NO:12 (CACGTAGAAACCCAGC), flanking the R434 target site (nt 371-388 and nt 537-554, respectively), and the lower primer was SEQ ID NO:13 (TAGATTATGGTTTCTGAGAACA), hybridizing in the E7 gene (nt 862-841). Standard PCR conditions were used (as supplied by Strategene, La Jolla, CA) with the following times and temperatures. The first strand cDNA was synthesized for 30 min at 45°C, followed by denaturation (92°C for 2 min) and 35 PCR cycles of: denaturation (92°C for 1 min), hybridization of primers (45°C for 45 sec) and polymerization (72°C for 1 min). This PCR amplification produced two products: an uncleaved product of 492 bp (amplified by SEQ ID NO: 11 and SEQ ID NO:13) and an internal control product of 326 bp (amplified by SEQ ID NO: 12 and SEQ ID NO:13). A control PCR reaction under the same conditions but with primers specific for an endogenous β-actin gene (SEQ ID NO:14: TGACGGGGTCACCCACACTGTGCCCATCTA, and SEQ ID NO:15:

CTAAGAAGCATTTGCGGTGGACGATGGAGGG) was used as a control to produce a band of 661 bp. Amplified products were separated on a 1.5% agarose gel and visualized with long-wave UV after ethidium bromide staining.

As shown in FIG. 6, all of the transfected cells produced the 661 bp β-actin control band. The control transfected (pCR16HH) cells and the inactive ribozyme construct (pCR16E6/E7RZi) transfected cells produced both the 492 bp and 326 bp products showing the presence of full-length E6/E7 transcripts, whereas the active ribozyme construct (pCR16E6/E7RZ) produced no detectable amount of the 492 bp band, indicating cleavage. These results support the finding that the decreased growth rate of the HKc transfected with the active ribozyme construct (pCR16E6/E7RZ) was due to the inhibition of E6/E7 gene expression.

Inactive Ribozyme Has *In Vivo* Antisense Activity

The effects of long-term expression of the E6/E7 genes with or without antisense containing ribozyme were investigated using transfected HKc as above, but maintained for up to 8 weeks in culture with standard cell culture medium after an initial G418 drug selection of four days. By the end of 8-week incubation, HKc transfected with a non-immortalizing gene (bacterial β-galactosidase) had senesced and detached. Therefore, mostly immortal cells are present in the cell cultures.

As shown in FIG. 7, cells transfected with the E6/E7 control (pCR16HH) were efficiently immortalized. Cells transfected with the active ribozyme construct (pCR16E6/E7RZ) showed little survival (about 10% relative to the control), as expected because of the limited E6/E7 gene expression. Cells transfected with the inactive ribozyme construct (pCR16E6/E7RZi) also showed decreased survival compared to the control, indicative that the antisense oligonucleotide portion of the construct, even in the absence of ribozyme activity, significantly inhibited E6/E7 gene expression. The lack of E6/E7 gene expression was confirmed using the RT-PCR assay which did not detect full length (492 bp) E6/E7 transcripts in either the cells transfected with active or inactive ribozyme constructs (pCR16E6/E7RZ or pCR16E6/E7RZi). These results also show the inhibitory activity *in vivo* of the antisense E6 oligonucleotide moiety of the pCR16E6/E7RZi construct.

To determine whether antisense oligodeoxynucleotides (ODN) complementary to HPV E6 could direct RNase-mediated degradation of the E6 mRNA, a ^{32}P-labeled synthetic RNA target from HPV-16 (nucleotides 413-446 of SEQ ID NO: 1) was produced by *in vitro* transcription and incubated with 2 units *E. coli* RNase H (Life Technologies) and 10 nmoles of ODN, which were synthesized in an Expedite 8900 DNA synthesizer using phosphoramidite chemistry (Perseptive Biosystems): Anti-E6 (5'-TTCTTCAGGACACAGT-3'; SEQ ID NO: 18, complement of SEQ ID NO: 3), M4 (5'-TTCTTCAGAGAACAGT-3'; SEQ ID NO: 7) or M7 (5'-TTCTTACTAGAACAGT-3'; SEQ ID NO: 19) at 37°C for up to 30 min in RNase H buffer (20 mM Tris-HCl, pH 8.0, 100 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 M dithiothreitol). Anti-E6 is complementary to nucleotides 430-445 of HPV-16 E6 mRNA. The oligomer 5'-GGCTTTTGACAGTTA-3' (SEQ ID NO: 20) is complementary to the HPV-16 sequence from nt 415-429 (SEQ ID NO: 2). The oligomer M4 and M7 differ from Anti-E6 by 4 and 7 bases, respectively (Table 1). Additional experiments used ^{32}P-labeled full-length HPV-16 E6/E7 genes (nt 97 to 868) under identical reaction conditions. Reactions were stopped with gel loading buffer (80% formamide, 0.01% bromphenol blue, 0.01% xylene cyanol) and heated at 65°C

for 5 min, then analyzed by electophoresis in 7 M urea 6% polyacrylamide gels. Gels were dried and exposed to Kodak BioMax BM radiographic film (Eastman Kodak, Rochester, NY).

Table 1. Synthetic ODN used. Mismatched bases are underlined.

Name	Sequence
Anti-E6	5'-TTC TTC AGG ACA CAG T-3'
M4	5'-TTC TTC AGA GAA CAG T-3'
M7	5'-TTC TTA CTA GAA CAG T-3'

Anti-E6 and M4 ODN were able to direct RNase H-mediated degradation of the target mRNA as shown by specific degradation of the transcript visualized by urea polyacrylamide gel electrophoresis, while the M7 mutant failed to direct RNase activity. Anti-E6 directed RNase H activity over the full length HPV-16 E6/E7 transcript; however, neither M4 or M7 affected this target. Thus, the Anti-E6 antisense ODN effectively directs RNase H activity over the entire HPV-16 E6/E7 mRNA.

The *in vivo* efficacy of single-stranded ODN can be seriously compromised by cellular exo- and endonucleases. To estimate the *in vivo* survival rate of HPV-16 antisense ODN, a 5'-fluorescein labeled Anti-E6 ODN (Genosys Biotechnologies, Inc.) was purified by high performance liquid chromatography (HPLC) and applied to HPV-16 cultured immortal (Hkc 16E6/E7-II) and cervical tumor cell lines (CasKi; ATCC CRL-1550 and SiHa; ATCC HTB-35) and to normal human keratinocytes (Hkc) from neonatal foreskins. HKc were cultured in keratinocyte-SFM (Life Technologies). CasKi cells were cultured in DMEM (Life Technologies) enriched with 5% fetal bovine serum (FBS, Gemini Bio-Products). Cells were cultured in 8-well slide chambers (Nunc, Inc., Naperville, IL) until 70% confluent in the appropriate media. Fresh medium containing 10 mM 5-fluorescein labeled ODN was added and cells were incubated for 2 hours at 37°C. Cultures were washed twice with phosphate buffered saline (PBS) and fresh medium was added. The slides were further incubated for various times (0-16 hours) at 37°C. ODN survival was estimated by the number of fluorescent cells detected with a Leitz Ortholux II fluorescence microscope using a FITC filter. The results are summarized in Table 2.

Table 2. Anti-E6 survival in different cell types. N, nuclear localization; C, cytoplasmic localization; ND, not detected; o/n, overnight.

Cell type	0 h	1h	2h	o/n
HKc	++++ N	++++ N	++++ N	++++ N
HKc16E6/E7II	++++ N	+++ NC	++ NC	+ NC
SiHa	++++ N	++ NC	++ NC	+ NC
CasKi	++++ N	+ N	ND	ND

The fluorescent label persisted for about 1 hour in tumor cell lines and for more than 2 hours in HPV-16 immortalized cell ines. No fluorescence remained after 12 hours. In contrast, normal keratinocytes retained the fluorescent label for 16 hours These results suggest that the stability of the Anti-E6 ODN correlated with the abundance of the target (HPV-16 E6). Thus, it appears that the efficiency of antisense inhibition is dependent on the steady state level of the ODN.

Modification of the chemical structure of therapeutic ODNs has been shown to effectively increase their half-life *in vivo* and to retain their capacity to direct RNase H activity on the target RNA (Yamaguchi et al., *Leukemia* 11:497-503, 1997; Temsamani et al., *J. Biol. Chem.* 266:468-472, 1991; Boiziau et al., *Biochimie* 73:1403-1408, 1991; Akhtar et al., *Life Sci.* 49:1793-1801, 1991; Agrawal et al., *Proc. Natl. Acad. Sci. U.S.A.*, 87:1401-1405, 1990). CasKi and QGU cervical tumor cells were cultured in 96-well plates and starved for 48 hours before treatment with 50 μM anti-E6 or M7 phosphorothioated ODNs (S-ODNs) for 72 hours. QGU cells (Shirasawa et al., *J. Gen. Virol.* 68:583-591, 1987) were cultured in F12/DMEM medium (Life Technologies). Anti-E6, M4 and M7 S-ODNs were obtained from Genosys Biotechnologies, purified by HPLC and applied to cells (50 μM) for 72 hours. ODN attrition was compensated by adding fresh ODN containing medium every 24 hours. Cell growth was estimated by the colorimetric quantification of Br-dUTP incorporation using the Cell Proliferation Kit III (Boehringer Mannheim, Indianapolis, IN). Growth inhibition of CasKi cells was observed only by the wild-type Anti-E6 ODN (Fig. 8). Untreated serum-deprived CasKi cells were used as a negative control. Similar results were obtained for QGU cells.

The antisense oligodeoxynucleotides (SEQ ID NO:6, SEQ ID NO:7) corresponding to the antisense sequences contained in the ribozymes (SEQ ID NO:4, SEQ ID NO:5) are synthesized as normal phosphodiester bond-linked oligonucleotides and phosphorothioate oligonucleotides to inhibit E6/E7 gene expression in human cells infected with HPV. The phosphorothioate antisense oligonucleotides (PS-oligonucleotides) are synthesized using standard methods (Agrawal et al., 1997, *Proc. Natl. Acad. Sci. USA* 94:2620-2625; Agrawal, 1996, *TIBTECH* 14:376-387). That is, the oligonucleotides are synthesized in which one of the non-bridging oxygens of the internucleotide phosphodiester linkages is replaced with sulfur; the synthesis is done using methods that produce a diastereomeric mixture of Rp and Sp PS-oligonucleotides. HKc that have been shown to contain HPV sequences or suspected of containing HPV sequences due to their source of origin (e.g., cervical cancer cells) are then transfected with about 25 nM to about 500 nM concentration, preferably about 200 nM concentrations, of oligonucleotides and PS-oligonucleotides, using lipofection as described above or any well known transfection methodology. Cells of the same origin as those transfected are used as a source of purified RNA, essentially as described above. Transfected cells are allowed to grow for 2-10 days in culture and then RNA is similarly isolated from them. E6 transcripts are assayed in the untransfected control cells and the antisense-oligonucleotide and antisense PS-oligonucleotide transfected cells using the RT-PCR assay substantially as described above. The amount of E6 transcript detected in transfected and untransfected cells of the same origin are compared and quantified.

In non-transfected control cells, E6 transcripts are present in most cells known to harbor HPV sequences and in most cervical cancer cells isolated from invasive cancers. In the matched cells for each sample transfected

with the antisense oligonucleotides and antisense PS-oligonucleotides, there is measurably less E6 transcript detected compared to the matched control. The inhibition of E6 gene expression ranges from about 10% to about 95%, depending on the combination of the cells used and the antisense oligonucleotide or PS-oligonucleotide. Some cells show about 70% to about 80% inhibition, whereas others show about 40% to about 50% inhibition, and still others show about 10% to about 25% inhibition of E6 gene expression compared to the matched untransfected control cells. In all cells, a positive control (e.g., β-actin gene expression (detected using the RT-PCR assay and primers of SEQ ID NO:14 and SEQ ID NO:15, as described above) varies by less than about 1% to about 5% from the HPV-antisense transfected cells and their matched untransfected control cells.

Similarly, antisense oligonucleotide and PS-oligonucleotide having sequences of SEQ ID NO:17 (TTCTTCAGAGAACAGTGGCTTTTGACAGTTA; corresponding to the antisense of the RNA sequence SEQ ID NO:16: UUCUUCAGAGAACAGUGGCUUUUGACAGUUA),representing the antisense DNA to nt 415 to 445 of HPV-16 are also synthesized using standard methods. Similarly the longer antisense oligonucleotide and PS-oligonucleotide are used in transfections of HKc, substantially as described above. The results of RT-PCR assays to determine the relative amounts of E6 transcripts in antisense oligonucleotide and PS-oligonucleotide transfected cells, compared to untransfected matched control cells, show somewhat less inhibition of E6 transcripts in the transfected cells compared to the smaller antisense oligonucleotide and PS-oligonucleotides described above. That is, about 0% to about 50% inhibition of E6 transcripts is detected in the tested cells. This lower level of inhibition may reflect less efficient transfection with the longer antisense oligonucleotide and PS-oligonucleotide.

When proliferation of the transfected cells (with any of the above-described antisense oligonucleotides and PS-oligonucleotides) and the matched untransfected control cells are compared using cell culture times of 1 to 5 days (starting at day 0, with about 10^6 cells/well of a standard 6-well culture plate and standard tissue culture conditions), the untransfected control cells generally show considerably more cell growth during the growth period than the matched transfected cells. The degree of proliferation inhibition is best seen at days 1 to 3, with some transfected cells showing approximately the same rate of cell proliferation as the matched control cells by day 5 after transfection. Some transfected cells show no inhibition of cell growth at any point in the testing period. Others show about 5% to about 40% inhibition of growth, depending on the cell line and the antisense oligonucleotide used. Retransfection at day 4, with those cells that initially show cell proliferation, reinstitutes the inhibition caused by the antisense oligonucleotides.

Similar inhibition results are obtained with antisense oligonucleotide sequences (SEQ ID NO:6, SEQ ID NO:7 and SEQ ID NO:17) synthesized as mixed backbone oligonucleotides, having 2'-*O*-methylnucleoside phophodiester bonds in place of phosphorothioate bonds in some positions, and oligonucleotides synthesized with ends of 2'-*O*-methylnucleosides, both types synthesized using known methods (Agrawal et al., 1997, *Proc. Natl. Acad. Sci. USA* 94:2620-2625). The mixed backbone antisense oligonucleotides, synthesized as racemic mixtures, are preferable to completely PS-oligonucleotides because of reduced toxicity to cells exhibited by the mixed backbone oligonucleotides.

Similar tests are performed with the antisense oligonucleotide sequences (SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:17) synthesized as analogs using standard methods. The analogs synthesized are methylphosphonates

(Miller et al., 1993, in *Antisense Research and Applications*, pp.189-203, Crooke & Lebleu, eds., CRC Press; Sarin et al., 1988, *Proc. Natl. Acad. Sci. USA* 85:7448-7451); phosphoramidites (Dagle et al., 1991, *Nucl. Acids Res.* 19(8):1805-1810; Froehler et al., 1988, *Nucl. Acids Res.* 16(11):4831-4839; Tanaka et al., 1987, *Nucl. Acids Res.* 15(15):6209-6224); phosphorodithioates (Marshall et al., 1992, *Proc. Natl. Acad. Sci. USA* 89:6265-6269), and N3'→P5'-phosphoramidites (Gryznov et al., *Nucl. Acids Res.* 24:1508-1514; Escude et al., 1996, *Proc. Natl. Acad. Sci. USA* 93(9):4365-4369; Chen et al., 1995, *Nucl. Acids Res.* 23(14):2661-2668). Analogs of the antisense oligoribonucleotides (SEQ ID NO:4, SEQ ID NO:5 and SEQ ID NO:16) are synthesized using known methods to produce oligoribonucleotide phosphorothioates (Agrawal et al., 1992, *Ann. New York Acad. Sci.* 660:2-10) and their 2'-*O*-alkyl analogs (Metelev et al., 1994, *Bioorg. Med. Chem. Lett.* 4:2929-2934; McKay et al., 1996, *Nucl. Acids Res.* 24:411-417; Monia et al., *J. Biol. Chem.* 268:14514-14522) and 2'-*O*-methylribonucleotide methylphosphonates (Kean et al., 1995, *Biochemistry* 34:14617-14620). All of the above-cited methods are known in the art and can readily be practiced by those skilled in the art; however, details regarding synthesis methods contained in the references cited herein are hereby incorporated by reference.

Antitumor Activity of Antisense Therapeutics in a Mouse Model

To determine whether antisense HPV-16 E6 ODN could inhibit the growth of cervical tumors *in vivo*, female C57 nude mice (3-4 weeks old) were injected subcutaneously with 5 x 10^6 CasKi cells where they produce solid tumors or ascites. When tumors were palpable, Alzet osmotic pumps model 1002 (Alza Corporation, Mountain View, CA) filled with 500 μg of Anti-E6 (SEQ ID NO: 18) or M7 (SEQ ID NO: 19) S-ODN in sterile water were implanted near the tumors for 14 days. Control animals received the tumor cells, but were treated with vehicle only. Anti-E6 inhibited the growth of these tumors, while M7 was ineffective. In M7 (SEQ ID NO: 19)-treated mice, tumor growth was identical to that observed with control animals. Thus, HPV-16 antisense oligonucleotides effectively inhibit cervical tumor cell growth in an *in vivo* animal model.

Other antisense oligonucleotides, antisense PS-oligonucleotides, mixed backbone antisense oligonucleotides, oligonucleotides having ends of 2'-*O*-methylnucleosides, and the oligonucleotide analogs or oligoribonucleotide analogs can also be tested in this mouse model. Typically, HPV-containing cervical tumor cells are injected into nude mice (e.g., subcutaneous at 10^6 to 10^8 cells/mouse). Preferred tumor cells are those isolated from spontaneous cervical carcinomas that are HPV positive (16 cell lines of this type are currently available for use). The mice are injected with 100 μg to 1,000 μg per day of the antisense therapeutic (i.p., s.c. or i.v.) before, simultaneously with or after injection of the human tumor cells (using protocols substantially as described in Skorski et al., *Proc. Natl. Acad Sci. USA*, 1997, 94(8):3966-3971). The mice are monitored for survival times and, using standard assay techniques, for production of solid tumors and/or ascites at daily to weekly intervals from 1 day to 5 months post-injection of the tumor cells.

Injection of the antisense therapeutics one day before or simultaneous with injection of the tumor cells shows that some of the antisense therapeutics have protective effects and prevent tumor development that otherwise occurs in the untreated controls injected with tumor cells. That is, treatment of mice with some of the antisense therapeutics entirely inhibits tumor cell growth or significantly slows growth of tumors or ascites compared to control

mice that receive the same number of injected tumor cells but no antisense treatment. For those antisense therapeutics that show antitumor activity in preliminary tests, daily injections of 100, 300 and 900 μg are subsequently tested to determine optimum antitumor activity and toxicity levels resulting from daily injections.

Injection of the antisense therapeutics at 1 day to 4 weeks after injection of the tumor cells shows that some of the antisense therapeutics are capable of limiting or eliminating tumors that otherwise grow in the untreated controls. Generally, antisense treatment is more effective, both in terms of long-term survival and inhibition of tumor cell proliferation, when treatment begins early after injection of tumor cells (i.e., within 1 to 2 weeks) and when mice receive repeated injections of the antisense therapeutic (e.g., daily or weekly dosages) during the test period. Also, the analogs (e.g., mixed backbone oligonucleotides and methylphosphonates, phosphoramidites, phosphorodithioates, or N3'→P5'-phosphoramidites) are generally more effective at lower concentrations (e.g., 100 μg to 500 μg/day) than the corresponding unmodified antisense oligonucleotides.

In other animal models, mice showing spontaneous growth of cervical cancers are treated by s.c. injection or painting of the cervical tumor with the antisense therapeutics described herein. Some tumors treated with some of the antisense therapeutics show a decrease in tumor growth or remission of the tumor. In general, the antisense therapeutics that are mixed backbone oligonucleotides or other analogs (e.g., methylphosphonates, phosphoramidites, phosphorodithioates, or N3'→P5'-phosphoramidites) are generally more effective at lower concentrations (e.g., 25 nM to 100 nM/day) than the corresponding unmodified antisense oligonucleotides.

Similarly, analogs of antisense oligoribonucleotides of SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:16, in which the analogs are oligoribonucleotide phosphorothioates, 2'-*O*-alkyl oligoribonucleotide phosphorothioates or 2'-*O*-methylribonucleotide methylphosphonates, are tested to antitumor activity in the assays described above. Some of the antisense oligoribonucleotide analogs also show significant antitumor activity *in vitro* and *in vivo*.

HPV-16 antisense molecules which inhibit cervical tumor cell growth in the mouse model discussed above are used for inhibition of human cervical tumor growth. In a preferred embodiment, for treatment of human cervical tumors, the cervix is injected daily with an antisense HPV-16 molecule such as Anti-E6 (SEQ ID NO: 18) S-ODN in a pharmaceutically acceptable carrier such as sterile PBS, in an amount between about 10 mg and 10,000 mg, more preferably between about 100 mg and 1,000 mg. Alternatively, the Anti-E6 (SEQ ID NO: 18) S-ODN is painted onto the cervix. The Anti-E6 S-ODN may also be applied to a ring of biocompatible material such as polypropylene or polyethylene, which is then placed around the cervix. These administration protocols all result in uptake of the ODN by the cervical tumor cells, inhibition of HPV-16, and inhibition of tumor cell growth.

While particular embodiments of the invention have been described in detail, it will be apparent to those skilled in the art that these embodiments are exemplary rather than limiting, and the true scope of the invention is defined by the claims that follow.

WHAT IS CLAIMED IS:

1. Analogs of antisense oligonucleotides comprising oligonucleotide sequences complementary to SEQ ID NO:2 or SEQ ID NO:3, wherein the analogs are phosphorothioate antisense oligonucleotides in which at least one phosphodiester bond is replaced with a phosphorothioate bond, mixed backbone antisense oligonucleotides in which at least one phosphodiester bond is replaced with a phosphorothioate bond and at least one phosphodiester bond is replaced with a 2'-*O*-methylnucleoside phophodiester bond, end-modified analogs in which at least one end has a 2'-*O*-methylnucleotide moiety, methylphosphonates, phosphoramidites, phosphorodithioates, or N3'→P5'-phosphoramidites.

2. Therapeutic compositions comprising any one of the analogs of antisense oligonucleotides of Claim 1, or mixtures thereof, in a pharmaceutically acceptable carrier.

3. Therapeutic compositions of Claim 2, further comprising a ribozyme containing sequences complementary to SEQ ID NO:2 or SEQ ID NO:3.

4. Analogs of antisense oligonucleotides comprising sequences of SEQ ID NO:6, SEQ ID NO:7 or SEQ ID NO:17, wherein the analogs are phosphorothioate oligonucleotides in which at least one phosphodiester bond is replaced with a phosphorothioate bond, mixed backbone oligonucleotides in which at least one phosphodiester bond is replaced with a phosphorothioate bond and at least one phosphodiester bond is replaced with a 2'-*O*-methylnucleoside phophodiester bond, end-modified oligonucleotides in which at least one end has a 2'-*O*-methylnucleotide moiety, methylphosphonates, phosphoramidites, phosphorodithioates, or N3'→P5'-phosphoramidites.

5. Therapeutic compositions comprising any one of the analogs of antisense oligonucleotides of Claim 4, or mixtures thereof, in a pharmaceutically acceptable carrier.

6. Analogs of antisense oligoribonucleotides of SEQ ID NO:4, SEQ ID NO:5 or SEQ ID NO:16, wherein the analogs are oligoribonucleotide phosphorothioates, 2'-*O*-alkyl oligoribonucleotide phosphorothioates or 2'-*O*-methylribonucleotide methylphosphonates.

7. Therapeutic compositions comprising any one of the antisense oligoribonucleotide analogs of Claim 6, or mixtures thereof, in a pharmaceutically acceptable carrier.

8. An antisense therapeutic composition comprising an oligonucleotide sequence of SEQ ID NO: 9.

9. A method of preventing transformation of a living cell by HPV comprising the steps of:
providing an antisense therapeutic composition according to Claim 8;
providing a living cell capable of being transformed by HPV;
transfecting said living cell with the antisense therapeutic composition; and
maintaining said living cell alive for sufficient time to inhibit expression of HPV gene E6.

10. The method of Claim 9, wherein the living cell is a human keratinocyte.

11. The method of Claim 9, wherein the living cell is a human cervical cell.

12. A method of preventing transformation of a living cell by HPV comprising the steps of:
providing one or more antisense oligonucleotides having a nucleotide sequence(s) complementary to SEQ ID NO:2 and/or SEQ ID NO:3;
providing a living cell capable of being transformed by HPV;

transfecting said one or more antisense oligonucleotides into the living cell; and

maintaining viability of the living cell for sufficient time to inhibit expression of HPV gene E6.

13. The method of Claim 12, wherein the living cell is a human keratinocyte.

14. The method of Claim 12, wherein the living cell is a human cervical cell.

15. A method of inhibiting expression of HPV gene E6 in a living cell comprising the steps of:

providing one or more antisense oligonucleotides having a nucleotide sequence(s) complementary to SEQ ID NO: 2 and/or SEQ ID NO: 3;

providing a biological sample comprising living cells capable of being infected with HPV;

transfecting said one or more antisense oligonucleotides into said living cells; and

maintaining the viability of said living cells for sufficient time to allow inhibition of HPV E6 gene expression to occur.

16. The method of Claim 15, further comprising repeating the transfecting and maintaining steps.

17. The method of Claim 15, wherein the living cells are human keratinocytes.

18. The method of Claim 15, wherein the living cells are human cervical cells.

19. The method of Claim 15, wherein the step of providing antisense oligonucleotides comprises administering said one or more antisense oligonucleotides to a living organism by subcutaneous, intraperitoneal or intravenous injection, or by painting said antisense oligonucleotides onto said biological sample *in situ*.

20. A method of inhibiting expression of HPV gene E6 in a living cell comprising the steps of:

providing one or more antisense oligonucleotide analogs having sequences of SEQ ID NO:6, SEQ ID NO:7, or SEQ ID NO:17, wherein the one or more antisense oligonucleotide analogs are phosphorothioate oligonucleotides in which at least one phosphodiester bond is replaced with a phosphorothioate bond, mixed backbone oligonucleotides in which at least one phosphodiester bond is replaced with a phosphorothioate bond and at least one phosphodiester bond is replaced with a 2'-*O*-methylnucleoside phophodiester bond, in which at least one end has a 2'-*O*-methylnucleotide moiety, methylphosphonates, phosphoramidites, phosphorodithioates, or N3'→P5'-phosphoramidites;

providing a biological sample comprising living cells capable of being infected with HPV;

transfecting said one or more antisense oligonucleotide analogs into said living cells; and

maintaining the viability of said living cells for sufficient time to allow inhibition of HPV E6 gene expression to occur.

21. A method for inhibiting expression of HPV gene E6 in a living cell comprising the steps of:

providing one or more antisense oligoribonucleotide analogs of oligoribonucleotides of SEQ ID NO:4, SEQ ID NO:5 or SEQ ID NO:16, wherein the one or more analogs are oligoribonucleotide phosphorothioates, 2'-*O*-alkyl oligoribonucleotide phosphorothioates or 2'-*O*-methylribonucleotide methylphosphonates;

providing a biological sample comprising living cells capable of being infected with HPV;

transfecting said one or more antisense oligoribonucleotide analogs into said living cells; and

maintaining the viability of said living cells for sufficient time to allow inhibition of HPV E6 gene expression to occur.

22. A method for inhibiting the growth of a cervical tumor, comprising the step of contacting said tumor with one or more analogs of antisense oligonucleotides comprising oligonucleotide sequences complementary to SEQ ID NO:2 or SEQ ID NO:3, wherein the one or more analogs are phosphorothioate antisense oligonucleotides in which at least one phosphodiester bond is replaced with a phosphorothioate bond, mixed backbone antisense oligonucleotides in which at least one phosphodiester bond is replaced with a phosphorothioate bond and at least one phosphodiester bond is replaced with a 2'-*O*-methylnucleoside phophodiester bond, end-modified analogs in which at least one end has a 2'-*O*-methylnucleotide moiety, methylphosphonates, phosphoramidites, phosphorodithioates, or N3'→P5'-phosphoramidites.

23. Use of one or more analogs of antisense oligonucleotides comprising oligonucleotide sequences complementary to SEQ ID NO:2 or SEQ ID NO:3, wherein the one or more analogs are phosphorothioate antisense oligonucleotides in which at least one phosphodiester bond is replaced with a phosphorothioate bond, mixed backbone antisense oligonucleotides in which at least one phosphodiester bond is replaced with a phosphorothioate bond and at least one phosphodiester bond is replaced with a 2'-*O*-methylnucleoside phophodiester bond, end-modified analogs in which at least one end has a 2'-*O*-methylnucleotide moiety, methylphosphonates, phosphoramidites, phosphorodithioates, or N3'→P5'-phosphoramidites, for treatment of a cervical tumor.

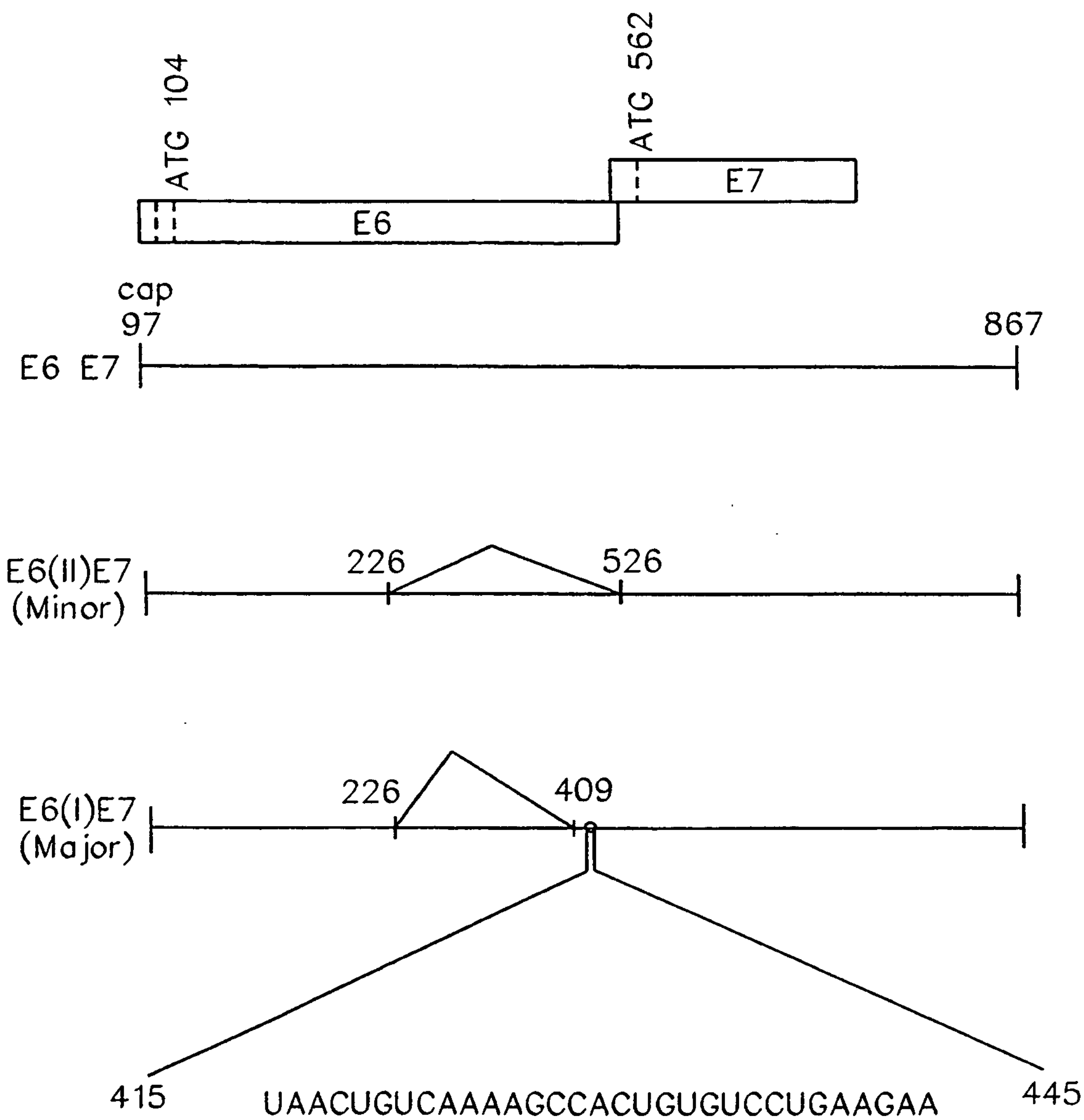
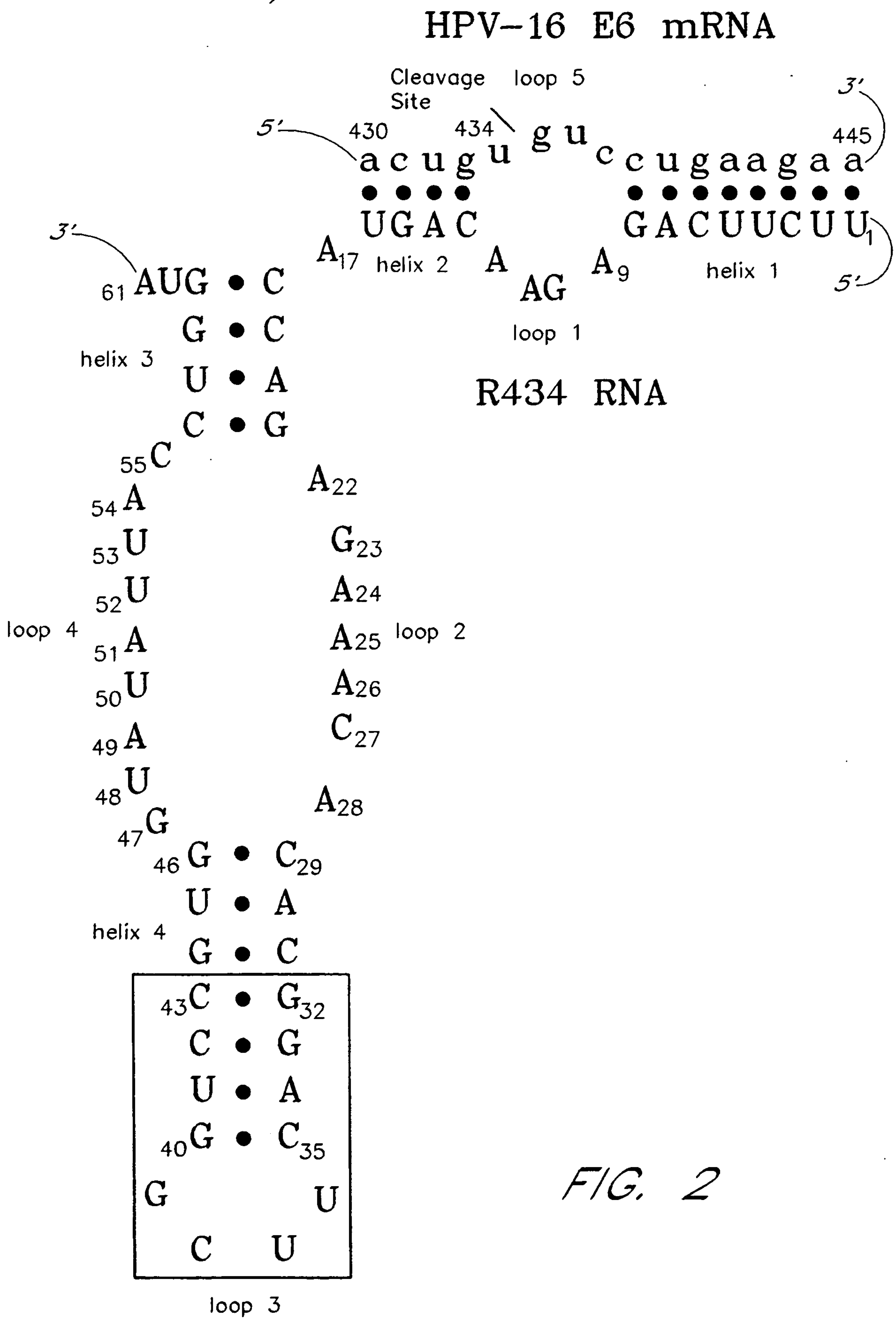

ATG 104
ATG 562
E6
E7
cap
97
867
E6 E7
E6(II)E7
(Minor)
226
526
E6(I)E7
(Major)
226
409
415
UAACUGUCAAAAGCCACUGUGUCCUGAAGAA
445

FIG. 1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Cleavage Site
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5'
3'
430
434
445
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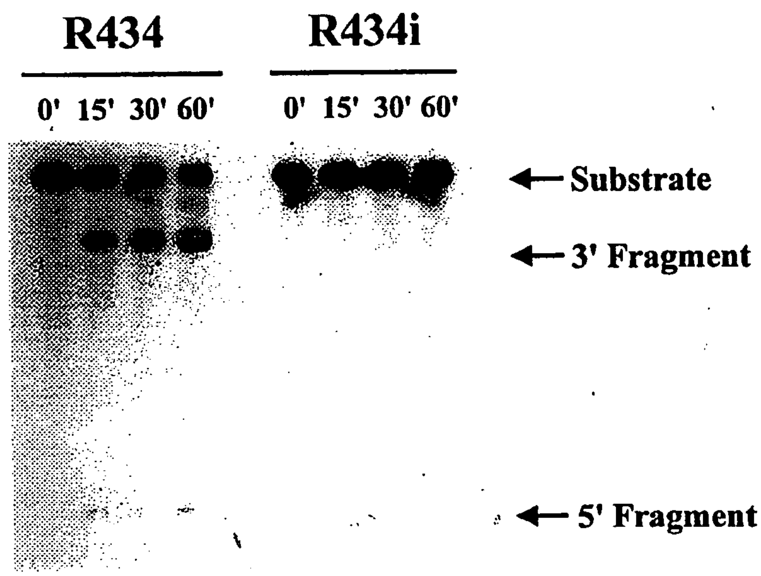
G A C U U C U U
A17
helix 2
A
AG
A9
helix 1
loop 1
R434 RNA
61 AUG • C
G • C
helix 3
U • A
C • G
55 C
54 A
53 U
52 U
loop 4
51 A
50 U
49 A
48 U
47 G
46 G • C29
U • A
helix 4
G • C
43 C • G32
C • G
U • A
40 G • C35
G
U
C
U
A22
G23
A24
A25
loop 2
A26
C27
A28
loop 3

FIG. 2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R434i
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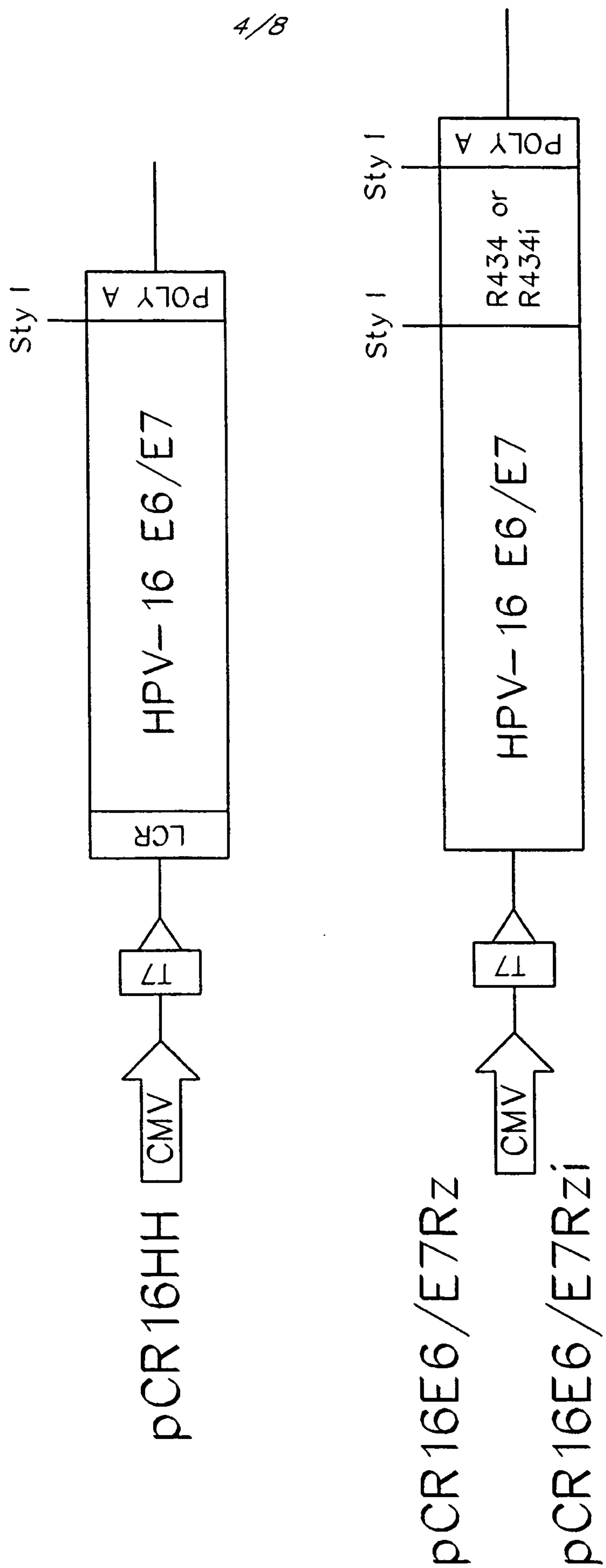
0' 15' 30' 60'
Substrate
3' Fragment
5' Fragment

FIG.3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T7
LCR
HPV-16 E6/E7
Sty I
POLY A
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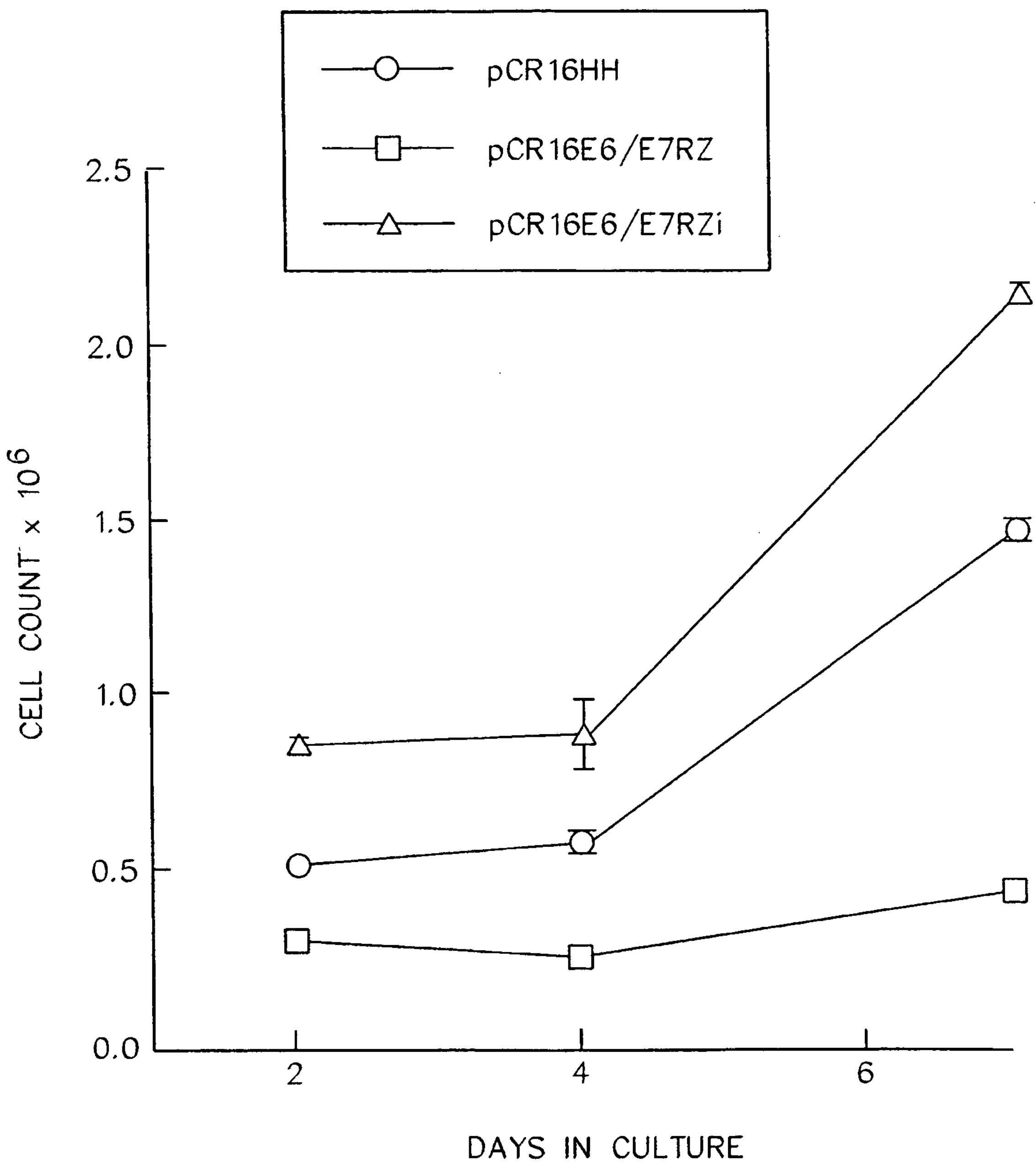
pCR16E6/E7Rzi
HPV-16 E6/E7
Sty I
R434 or R434i
Sty I
POLY A

FIG. 4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pCR16E6/E7RZ
pCR16E6/E7RZi
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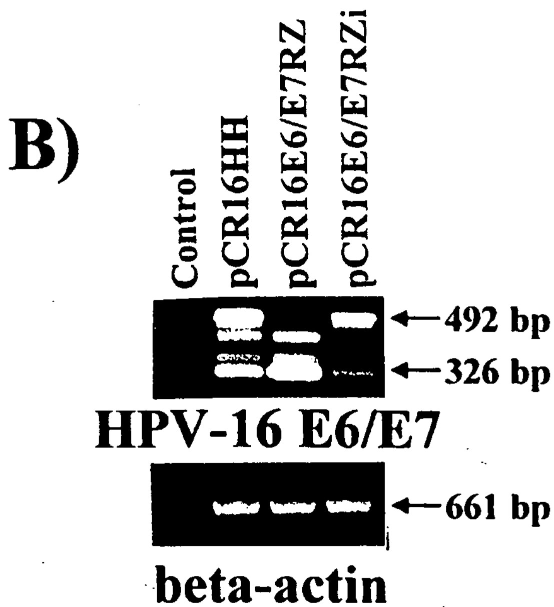
2
4
6
DAYS IN CULTURE

FIG. 5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pCR16E6/E7RZi
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HPV-16 E6/E7
661 bp
beta-actin

FIG. 6

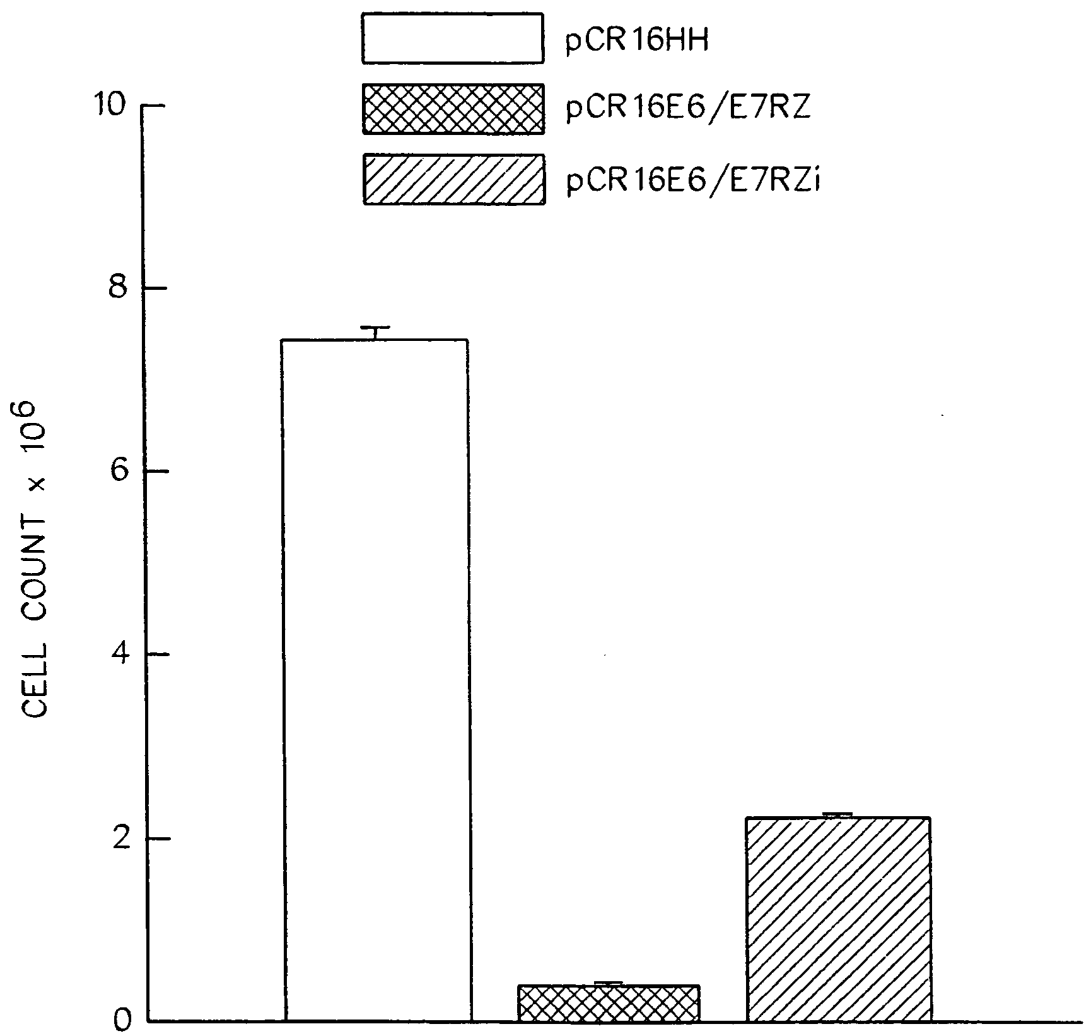
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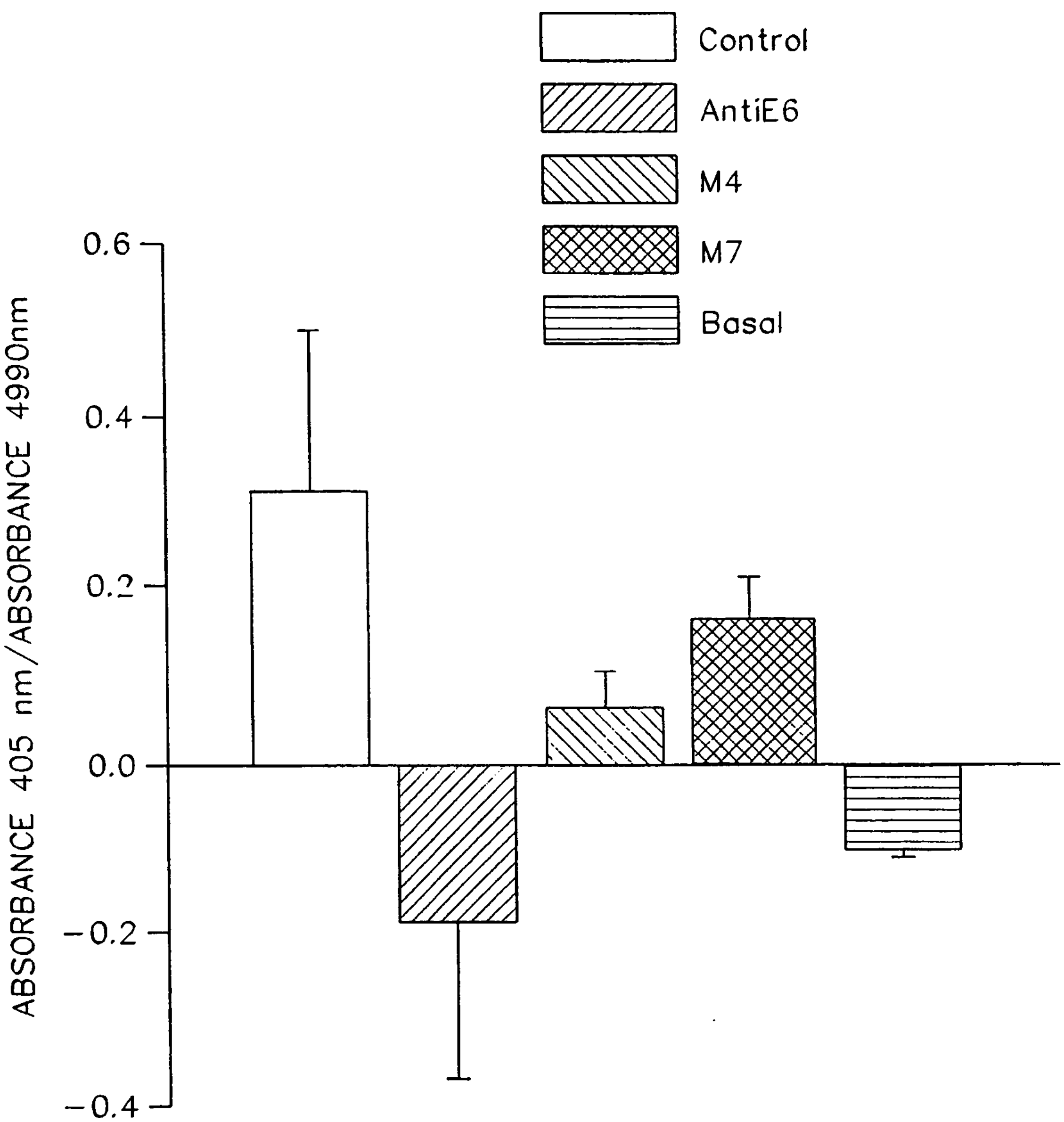
FIG. 7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M4
M7
Basal
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FIG. 8

SEQUENCE LISTING

<110> Government of the United States of America,
As Represented by the Secretary, Department
of Health and Human Services
DiPaolo, Joseph
Alvarez-Salas, Luis

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<151> 1997-09-05

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/18320

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/11 C07H21/00 A61K31/70 //C07K14/025,C12N9/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07H A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 95 31552 A (US HEALTH ;NORTHERN ILLINOIS UNIVERSITY (US); HAMPEL ARNOLD (US);) 23 November 1995 see abstract see page 20, line 1 - line 5 see claims 1,2 seq. IDs 3,6,8,11 ---	1-23
Y	WO 93 20095 A (ISIS PHARMACEUTICALS INC ;CROOKE STANLEY T (US); MIRABELLI CHRISTO) 14 October 1993 see abstract see page 24, line 17 - line 23 see example 3 see claims 1-9 --- -/--	1-23

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 February 1999	02/03/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Galli, I

INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/US 98/18320

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WO 95 13833 A (GENTA INC) 26 May 1995 see abstract see examples 1-27 see examples A-H	1-23
A	WO 96 39501 A (HOFFMANN LA ROCHE ;HYBRIDON INC (US)) 12 December 1996 see abstract see table 1B see example 5	1-23

INTERNATIONAL SEARCH REPORT	...ternational application No. PCT/US 98/ 18320

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 19, 22, 23 and (as far as methods in vivo are envisaged) claims 9-18,20,21 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 98/18320

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